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Research

Evolution of selenium hyperaccumulation in *Stanleya* (Brassicaceae) as inferred from phylogeny, physiology and X-ray microprobe analysis

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Summary

• Past studies have identified herbivory as a likely selection pressure for the evolution of hyperaccumulation, but few have tested the origin(s) of hyperaccumulation in a phylogenetic context. We focused on the evolutionary history of selenium (Se) hyperaccumulation in *Stanleya* (Brassicaceae).

• Multiple accessions were collected for all *Stanleya* taxa and two outgroup species. We sequenced four nuclear gene regions and performed a phylogenetic analysis. Ancestral reconstruction was used to predict the states for Se-related traits in a parsimony framework. Furthermore, we tested the taxa for Se localization and speciation using X-ray microprobe analyses.

• True hyperaccumulation was found in three taxa within the *S. pinnata/bipinnata* clade. Tolerance to hyperaccumulator Se concentrations was found in several taxa across the phylogeny, including the hyperaccumulators. X-ray analysis revealed two distinct patterns of leaf Se localization across the genus: marginal and vascular. All taxa accumulated predominantly (65–96%) organic Se with the C–Se–C configuration.

• These results give insight into the evolution of Se hyperaccumulation in *Stanleya* and suggest that Se tolerance and the capacity to produce organic Se are likely prerequisites for Se hyperaccumulation in *Stanleya*.

Introduction

Elemental hyperaccumulation is an intriguing trait that has been documented in over 500 plant species (Krämer, 2010; Cappa & Pilon-Smits, 2014). The criterion for a species to be a hyperaccumulator depends on the element in question and ranges from 0.01% to 1% DW (Krämer, 2010). This criterion has been applied to several toxic elements including arsenic (As), cadmium (Cd), cobalt (Co), lead (Pb), nickel (Ni), selenium (Se) and zinc (Zn) (Krämer, 2010). There is convincing evidence for several adaptive advantages of elemental hyperaccumulation, including protection from herbivores (Pollard & Bakers, 1997; Boyd & Moar, 1999; Jhee *et al.*, 1999; Freeman *et al.*, 2007, 2009; Quinn *et al.*, 2008, 2010), protection from pathogens (Boyd *et al.*, 1994; Hanson *et al.*, 2004) and elemental allelopathy to neighboring plants (El Mehdawi *et al.*, 2011). Given that these elements are toxic in high enough concentrations to both plants and their associated herbivores and pathogens, it is reasonable to hypothesize that only those genotypes that can survive with high internal concentrations of these toxic elements or exclude them altogether, will have a selective advantage in these environments.

Relatively few studies have tested the number of origins of hyperaccumulation in a phylogenetic context. From mapping the occurrence of hyperaccumulators on the angiosperm phylogeny (Cappa & Pilon-Smits, 2014) it is clear that hyperaccumulators constitute a polyphyletic group across flowering plants. Interestingly, over half of hyperaccumulators are found in three orders: Malpighiales (eight families and 127 taxa), Brassicales (two families and 102 taxa) and Asterales (three families and 79 taxa). The Brassicaceae constitute the largest fraction of known hyperaccumulators for any family, with >100 taxa. Krämer (2010) proposed at least 13 independent origins of hyperaccumulation within the Brassicaceae. Even within a genus there can be multiple origins of tolerance or hyperaccumulation. For example, Cecchi *et al.* (2011) suggested at least six origins of obligate endemics to serpentine soils in the genus *Onosma* (Boraginaceae) with nonserpentine endemics representing the ancestral phenotype. *Alyssum* (Brassicaceae) has been shown to also have multiple origins of Ni hyperaccumulation, with multiple events of local adaptation and selection across southern European serpentine soils (Mengoni *et al.*, 2003; Cecchi *et al.*, 2010).

We are particularly interested in the evolution and origin of Se tolerance and hyperaccumulation in *Stanleya* Nutt. (Brassicaceae; tribe Thelypodieae). *Stanleya* comprises seven species and one of these, *S. pinnata*, is divided into three varieties. Treatment of these varieties differs depending on the flora used: Holmgren *et al.* (2005) recognized var. *integrifolia*, var. *inyoensis* and var. *pinnata*, whereas Al-Shehbaz (2010) recognized var. *integrifolia*, var. *pinnata* and var. *texana*. All seven species occur only in the western US; for ranges see Cappa *et al.* (2014). *Stanleya pinnata* var. *pinnata* is well-documented as an Se hyperaccumulator (Beath *et al.*, 1939a,b, 1940, 1941; Feist & Parker, 2001; Galeas *et al.*, 2007; Freeman *et al.*, 2010) and occurs in most western states. *Stanleya bipinnata* has also been reported to be an Se hyperaccumulator (Beath *et al.*, 1940).

Selenium hyperaccumulation is particularly interesting because, while Se is an essential micronutrient for many animals, prokaryotes and algae, it has not been shown to be essential for vascular plants (Ellis & Salt, 2003; Sors *et al.*, 2005; Zhang & Gladyshev, 2008). Most plants likely take up Se inadvertently because it is atomically similar to sulfur (S). For a plant to be considered an Se hyperaccumulator it must accumulate Se to $>1000\ mg\ kg^{-1}$ or 0.1% of DW (Krämer, 2010). *Stanleya pinnata* var. *pinnata* can accumulate Se up to 0.5% DW and preferentially takes up Se over S (Parker *et al.*, 2003; White *et al.*, 2007; Harris *et al.*, 2014). Below the threshold of Se hyperaccumulation, two other tiers of Se accumulation can be distinguished: Se accumulators/secondary Se accumulators accumulate 0.01–0.1% Se in the field (100–1000 $mg\ kg^{-1}$ DW), while non-Se accumulators accumulate $<0.01\%$ ($100\ mg\ kg^{-1}$ DW) (El Mehdawi & Pilon-Smits, 2012).

In trying to understand the evolution of Se hyperaccumulation, key questions to address are: why do plants hyperaccumulate this nonessential, toxic element (i.e. which selection pressures may have led to Se hyperaccumulation)? As mentioned above, protection from herbivores and pathogens may have been a selection pressure for Se hyperaccumulation, in addition to benefits from elemental allelopathy. A second question is: how do plants hyperaccumulate Se? Finally, which genetic and metabolic changes have occurred that led to the evolution of Se hyperaccumulation? Increased, constitutive expression of sulfate transporters may be one of the mechanisms of Se hyperaccumulation. Freeman *et al.* (2010) showed that several transcripts for sulfate transporters were constitutively upregulated in the Se hyperaccumulator *S. pinnata* relative to nonhyperaccumulator *S. albescens*. There is also evidence for the presence of sulfate-transporter homologs with enhanced selenate specificity (Harris *et al.*, 2014). The currently hypothesized tolerance mechanism of Se hyperaccumulation is the production of the organic selenocompound selenocystathionine (SeCyst) and methyl-SeCys. All plants can assimilate inorganic selenate into selenocysteine (SeCys) via the sulfate assimilation pathway (Terry *et al.*, 2000). This SeCys is toxic when it is nonspecifically incorporated into proteins. This toxicity can be prevented if the SeCys is further metabolized to selenocystathionine (SeCyst), selenomethionine (SeMet) or methyl-SeCys (Neuhierl & Böck, 1996; Sors *et al.*, 2005; Freeman *et al.*, 2006).

The primary question addressed in this study is: what is the evolutionary history of Se hyperaccumulation in *Stanleya*? To address this question we used a combination of physiological, molecular and biophysical approaches. We determined Se tolerance across *Stanleya* taxa in a sterile common-garden environment. We also determined Se distribution and chemical speciation in vegetative and reproductive tissues, using X-ray microprobe analyses. Furthermore, we used a combination of molecular and morphological traits to resolve the phylogenetic relationships of *Stanleya*. Finally, we mapped Se tolerance and Se accumulation properties onto the inferred phylogenetic relationships of *Stanleya*. By mapping these Se-related traits onto the *Stanleya* phylogeny we show hyperaccumulation evolved in the *S. bipinnata/pinnata* clade and hypothesize tolerance likely preceded hyperaccumulation in *Stanleya*.

Materials and Methods

Sampling

All taxa included in this study were collected from field sites determined from herbaria databases. When possible, we collected a minimum of three populations and a minimum of three individuals from those populations for each taxon (for details see Cappa *et al.*, 2014). At each site, leaves, seeds and soil were collected. The leaves were silica-dried (Chase & Hills, 1991) and later used for DNA extraction and molecular phylogenetic analyses (as described below). Seeds were used for the common garden experiment and XAS analyses (as described below).

Plant growth

Seeds were surface sterilized; rinsed in 70% ethanol for 30 s, placed on a rocker for 20 min in 15% bleach and rinsed five times with sterile distilled deionized H_2O. The seeds were placed at 4°C for 48 h before being transferred to sterile Petri dishes with filter paper. Once cotyledons emerged, the seedlings were transferred to ½ Murashige & Skoog (1962) agar containing 0, 20, 53, 80 or 160 μM sodium selenate ($NaSeO_4$). The plants were grown for 30 d at 23°C in a growth chamber at a light intensity of 150 μmol with a 16 h : 8 h, light : dark period. The 53-μM-treated plants were used for XAS analysis. Due to low germination rates of *S. bipinnata* and *S. tomentosa* they were only grown in the 0, 53 and 80 μM $NaSeO_4$ treatments.

Tolerance and accumulation analysis

Whole plants were harvested and roots rinsed in deionized H_2O to remove any external Se. Plants were dried at 50°C for 72 h before being weighed and nitric acid digested according to Zarcinas *et al.* (1987). The digest was analyzed via inductively coupled plasma atomic emission spectroscopy (ICP-AES) according to Fassel (1978). Pearson's correlation was used to test for significant correlations between internal Se concentration and biomass, as a measure of tolerance. This parameter was chosen because it best reflects Se tolerance. Growth inhibition in response to external Se

concentrations would reflect Se resistance, which could also be due to exclusion. ANOVA followed by Tukey–Kramer *post hoc* analyses were carried out to test for significant differences in Se accumulation between species. Both analyses were conducted in R (v2.15.1; www.r-project.org). Graphs were produced in SigmaPlot v11 (Systat Software, San Jose, CA, USA).

X-ray microprobe analysis

Frozen intact mature leaves and field collected seeds were analyzed via μX-ray fluorescence (XRF) for chemical mapping of Se, Ca and Fe. Micro X-ray near edge absorption spectrometry (XANES) was used to determine the chemical speciation of Se using standard selenocompounds XANES spectra (Quinn *et al.*, 2011). The spectra were fitted using a linear least-squares combination where the quality of the fit was measured as the sum of squares.

DNA extraction and amplification

Total genomic DNA was isolated from silica-dried tissue using a Qiagen DNeasy Kit according to manufacturer's instructions or the protocol described by Alexander *et al.* (2007). The following four nuclear markers were used: chalcone synthase (CHS; Koch *et al.*, 2000), luminidependens (LD; Slotte *et al.*, 2006), internal transcribed spacer (ITS; Blattner, 1999) and SATF - 5′ AGAT GTTTTCTTGGAAATATTATCAG 3′, SATR - 5′ TTAATG RTCAAGAATATTAGATCAAAC 3′ (SAT, developed for this study). At least three individuals per taxon (when possible) were sequenced for all four gene regions. All four gene regions were amplified on a thermocycler according to the following temperature regime: 96°C for 3 min (initial denaturation) followed by 10 cycles of 96°C for 45 s (denaturation), 50°C for 30 s (annealing) and 72°C for 2 min (extension), then 25 cycles of 96°C for 20 s, 50°C for 30 s and 72°C for 2 min. Polymerase chain reaction (PCR) products were purified with a Qiagen PCR Purification Kit and the resulting purified products were sequenced by the University of Chicago Cancer Research Center DNA Sequencing Facility via ABI DNA Analyzers. *Arabidopsis thaliana* and *Brassica rapa* sequences were obtained from TAIR and GenBank, respectively. Leaf samples from herbarium specimens were generously donated by four herbaria (MO, NY, OSU, RM) for two *S. bipinnata*, two *S. pinnata* var. *texana*, one *Thelypodium laciniatum*, one *Thelypodiopsis ambigua* and all four *S. confertiflora* samples (Supporting Information Table S1). Primers used for amplification were also used for sequencing. All sequences were deposited in GenBank (Table S1).

Phylogenetic analyses

Preliminary nucleotide alignments were obtained independently for each gene region using MAFFT v6.5 (Katoh & Toh, 2008). G-INS-i, the most accurate MAFFT algorithm for aligning gene regions other than rDNA, was used for all four gene regions. The 1PAM nucleotide scoring matrix and the default gap opening penalty (1.53) were applied. Manual adjustments to the MAFFT alignments were performed in MacClade v4.08 (Maddison & Maddison, 2001) using the procedure outlined by Simmons (2004) following Zurawski & Clegg (1987). Two ambiguously aligned regions were eliminated from the analysis (LD, 271–281;

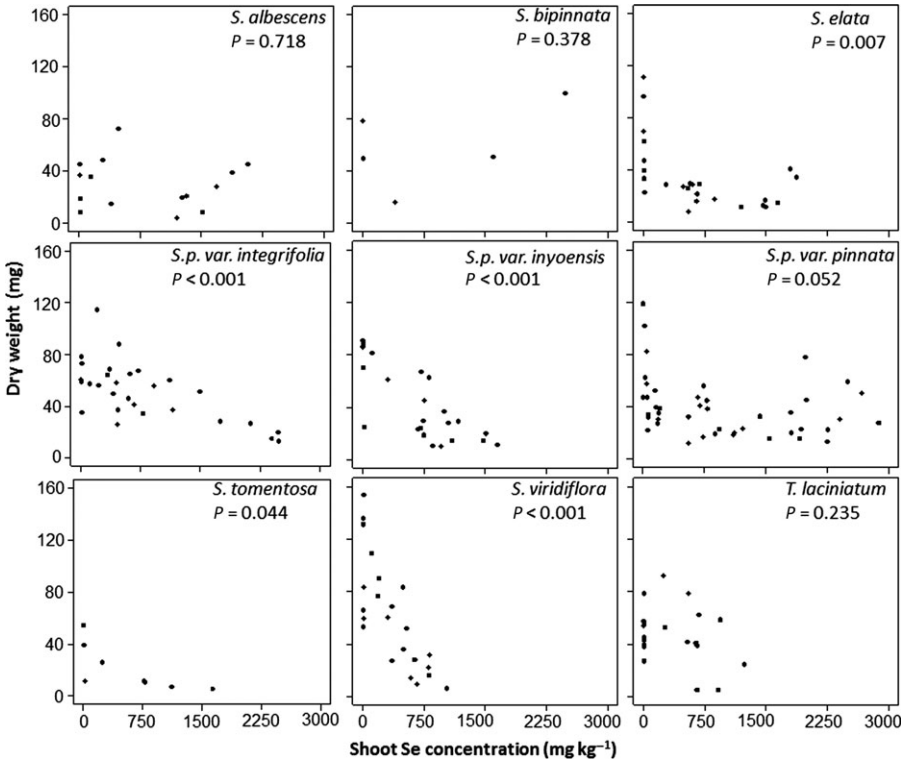

Fig. 1 Selenium (Se) tolerance of *Stanleya albescens*, *S. bipinnata*, *S. elata*, *S. pinnata* var. *integrifolia*, *S. pinnata* var. *inyoensis*, *S. pinnata* var. *pinnata*, *S. tomentosa*, *S. viridiflora* and *Thelypodium laciniatum*, as judged from their dry weight production as a function of their internal Se concentration when grown on agar medium supplemented with 0, 20, 80 or 160 μM of sodium selenate (pooled data). A P-value < 0.05 indicates a significant positive or negative correlation between internal Se concentration and growth (Pearson's correlation analysis).

SAT, 457–467). Ambiguously aligned nucleotides of individual sequences in regions that could not be unambiguously aligned with the remaining sequences were re-scored as ambiguous ('?').

Gap characters, whose inclusion often affects the inferred tree topology and increase branch-support values (Simmons *et al.*, 2001), were manually scored using modified complex indel coding (Simmons & Ochoterena, 2000; Müller, 2006). A total of 19 parsimony-informative gap characters were scored from unambiguously aligned regions (ITS, 4; LD, 7; SAT, 8).

A total of 15 vegetative and reproductive morphological characters (Table S2), were included in the simultaneous analysis. These characters were chosen based on the Flora of North America (Al-Shehbaz, 2010) dichotomous key.

Equally weighted parsimony tree searches were conducted using TNT v1.1 January 2013 (Goloboff *et al.*, 2008). Branches with a minimum possible optimized length of zero were collapsed to improve efficiency of tree searches and help minimize artifacts caused by missing data (Kitching *et al.*, 1998; Davis *et al.*, 2005). Up to 50 trees were held (Davis *et al.*, 2005) within each of 10 000 random addition sequence (RAS) tree bisection reconnection (TBR) searches. Parsimony jackknife (Farris *et al.*, 1996) analyses were conducted with the removal probability set to *c.* e^{-1} (0.37). One thousand parsimony jackknife replicates were performed with 100 RAS TBR searches (each with a maximum of 50 trees held) per replicate.

jModeltest v2.1.4 (Posada, 2008) was used to select the best-fit likelihood model for each data matrix using the Akaike Information Criterion (AIC; Akaike, 1974) without considering invariant-site models following Yang (2006). The models selected all incorporate the gamma distribution (Yang, 1993). The Q-matrices selected are HKY (SAT), SYM (ITS), TIM2 (CHS), TPM1uf (LD) and GTR (all four gene regions together).

PartitionFinder v1.1.1 (Lanfear *et al.*, 2012) was used to determine the number of partitions to use for the all-four-gene-regions analysis by using the AIC and the defaults for all other settings. PartitionFinder selected a different partition for each of the four gene regions. This partitioning scheme was implemented in GARLI v2.01.1067 (Zwickl, 2006) by allowing different model parameters and different subset rates between the four partitions

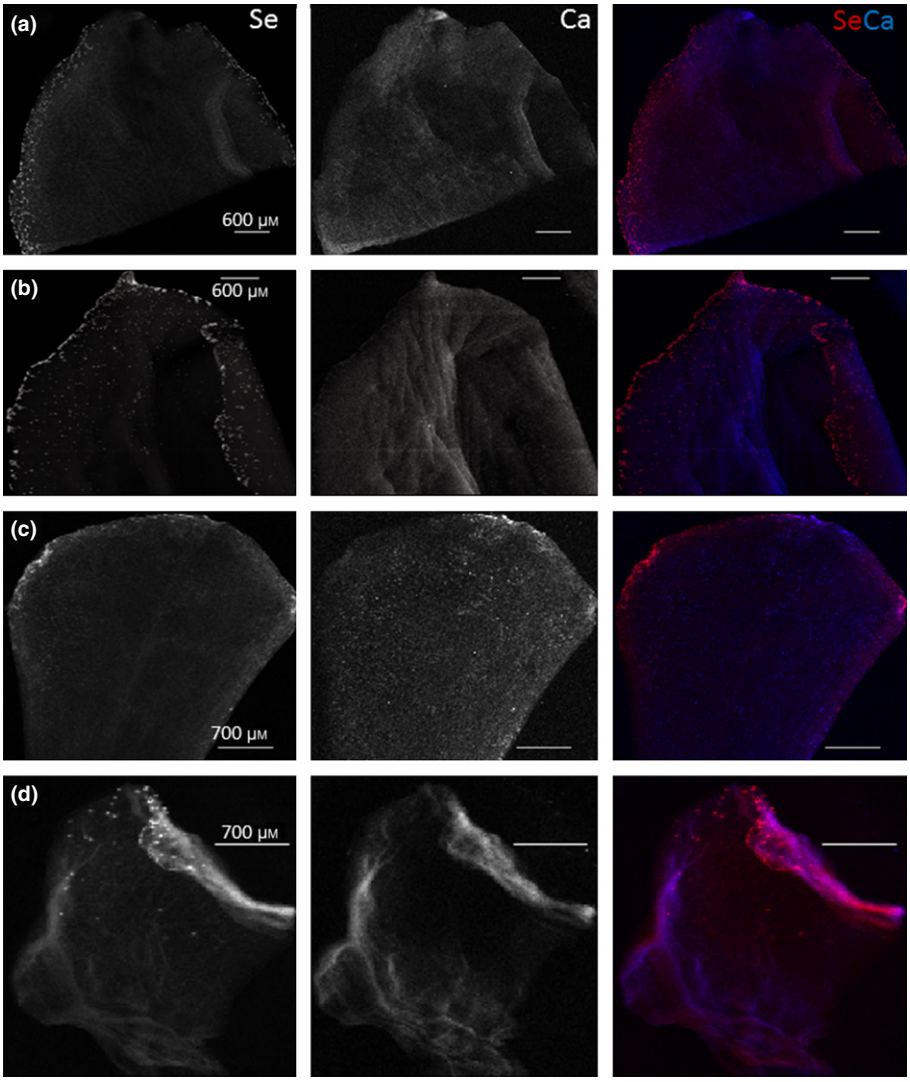

Fig. 2 X-ray fluorescence elemental mapping of leaves of different *Stanleya* species grown on agar medium supplemented with 53 μM sodium selenate. (a) *S. pinnata* var. *integrifolia*; (b) *S. pinnata* var. *pinnata*; (c) *S. bipinnata*; (d) *S. tomentosa*. Left column, selenium (Se, in white); middle column, calcium (Ca, in white); right column, Se (red) and Ca (blue) overlay.

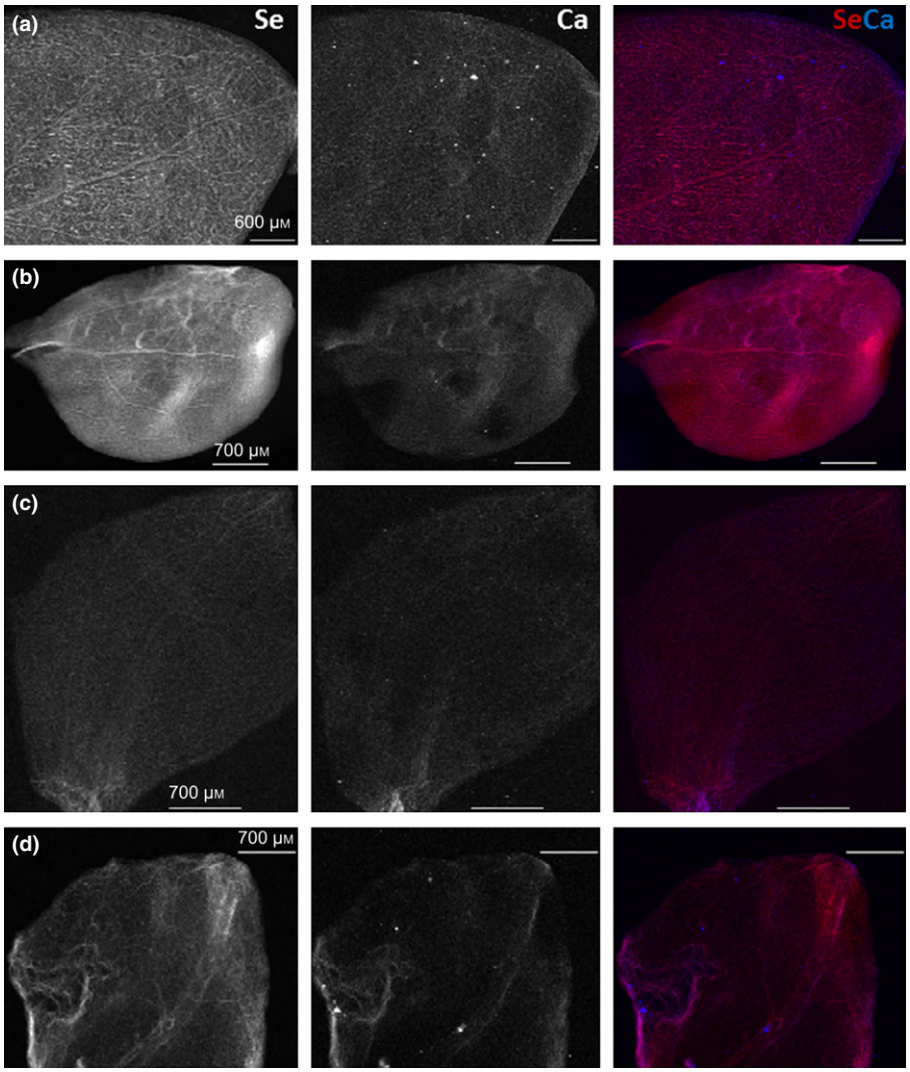

Fig. 3 X-ray fluorescence elemental mapping of leaves of different *Stanleya* and *Thelypodium* species grown on agar medium supplemented with 53 μM sodium selenate. (a) *S. pinnata* var. *inyoensis*; (b) *S. viridiflora*; (c) *S. elata*; (d) *Th. laciniatum*. Left column, selenium (Se, in white); middle column, calcium (Ca, in white); right column, Se (red) and Ca (blue) overlay.

(linkmodels = 0; subsetspecificrates = 1) with the GTR + Γ model for each partition.

Maximum likelihood analyses (Felsenstein, 1973) were performed with GARLI. Because the TIM and TPM Q-matrices are not implemented in GARLI, the GTR model was applied to CHS and LD instead. ITS was analyzed using the SYM model by setting all nucleotides to equal frequencies and SAT was analyzed using the HKY model. Following the recommended setting in GARLI, branches with a length of 1×10^{-8} (i.e. effectively zero; Zwickl, 2012) were collapsed. The GARLI analyses were performed by using the least rigorous settings for an intensive search recommended by Zwickl (2009; streefname = stepwise; attachmentspertaxon = 50, genthreshfortopoterm = 20 000, numberofprecreductions = 20, treerejectionthreshold = 100) for both optimal-tree searches (1000 search replicates) and the bootstrap (BS; Felsenstein, 1985; 1000 replicates, each with 10 searches).

Ancestral reconstruction

All weakly supported branches, with < 50% jackknife and bootstrap support, were collapsed in TreeGraph2 v2.0.44 (Stöver & Müller, 2010). The resulting tree was imported into Mesquite v2.75 (Maddison & Maddison, 2011) where a categorical character matrix was created for accumulation and tolerance. Accumulation was designated as follows: nonhyperaccumulator < 1000 and hyperaccumulator > 1000 mg Se kg^{-1} DW. For these designations, we used the highest Se concentration recorded for a given taxon published from field surveys (Beath *et al.*, 1939b, 1940, 1941; Cappa *et al.*, 2014). The reason why the maximum reported field Se concentration was used as the measure for Se accumulation property, rather than values from our agar experiment, is that we consider it more reliable than Se accumulation potential in an artificial setting. Published studies have shown that plants known to not hyperaccumulate in the field (e.g. *Brassica juncea*, *Arabidopsis thaliana*) can reach tissue Se concentrations above 0.1% DW in artificial systems (Pilon-Smits *et al.*, 1999; Zhang *et al.*, 2007). Tolerance was scored from the scatterplots as the 50% inhibition point calculated from biomass production as a function of internal Se concentration using the linear equation produced (Fig. 1). Tolerance was categorically ranked as follows: nontolerant < 1000 and tolerant > 1000 mg Se kg^{-1} DW. *B. rapa* and *A. thaliana* were scored as

Se tolerant (Garifullnia *et al.*, 2003; Van Huysen *et al.*, 2003) and Se sensitive (Zhang *et al.*, 2007), respectively. The accumulation and tolerance characters were mapped onto the tree using Fitch (1971) optimization in Mesquite.

Results

Tolerance and accumulation

As a measure of tolerance we plotted the internal Se concentration attained in the common-garden agar experiment (Fig. S1) against the total biomass for each individual across all treatments: 0, 20, 80 and 160 μM (Fig. 1). *Stanleya pinnata* var. *pinnata* reached the highest maximum tissue Se concentrations (close to 3000 mg Se kg^{-1} DW) followed by *S. pinnata* var. *integrifolia* and *S. bipinnata*. *Thelypodium laciniatum* and *S. viridiflora* had the lowest Se accumulation amounts (Fig. 1). *Stanleya bipinnata* is the only species that had a trend for increasing biomass production with increasing tissue Se concentration, but due to low germination there were too few plants to show a significant effect. In *S. albescens* and *Th. laciniatum* internal Se concentration and biomass were not significantly correlated; the same was true for *S. pinnata* var. *pinnata*, although the *P*-value (0.052) was close to significance. *Stanleya tomentosa* had a marginally significant negative response to Se concentration, and *S. elata*, *S. pinnata* vars *integrifolia* and *inyoensis* and *S. viridiflora* had a significantly negative response to increasing internal Se concentration ($P < 0.01$).

XRF

Two distinct patterns of Se localization were found in *Stanleya*. Four of the eight species (*S. bipinnata*, *S. pinnata* var. *integrifolia*,

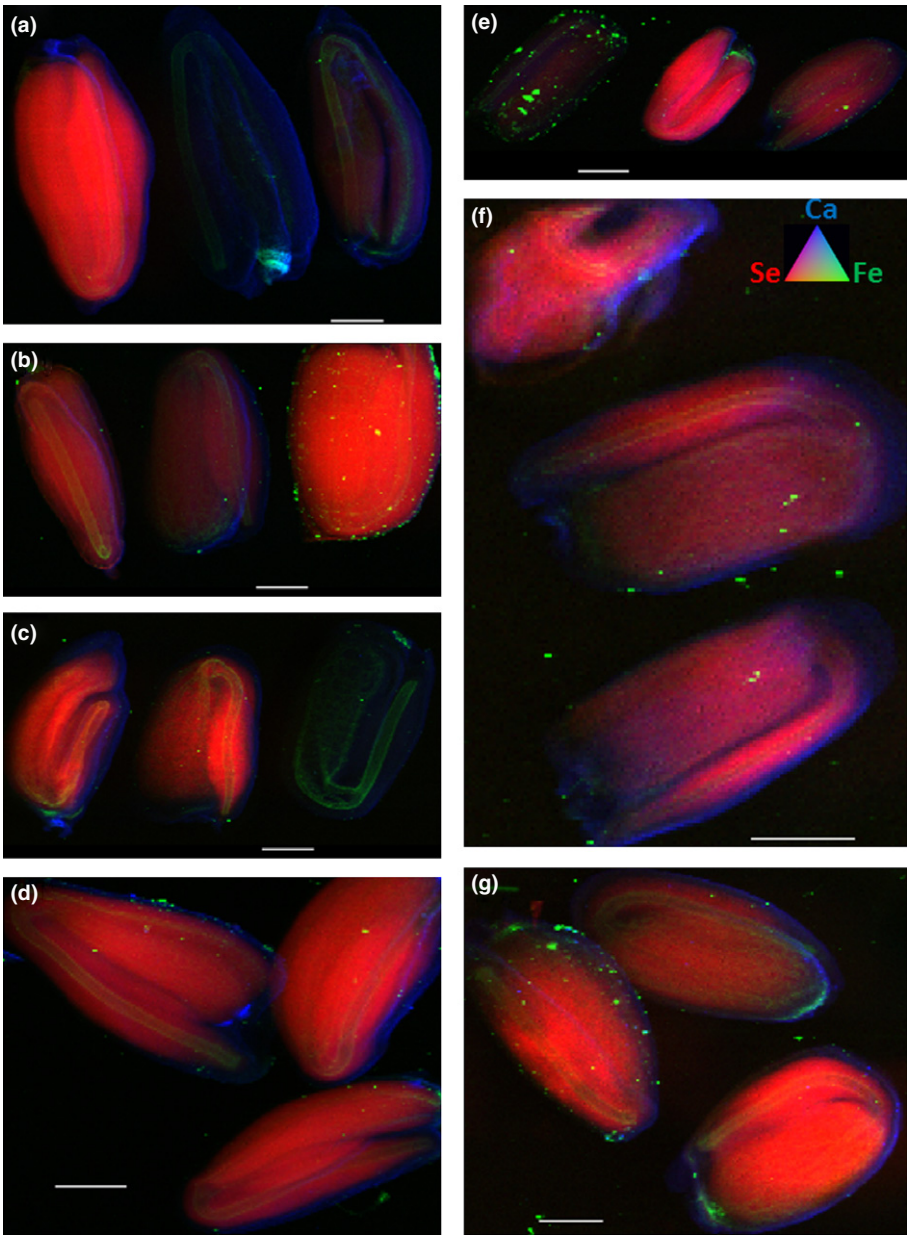

Fig. 4 X-ray-fluorescence elemental mapping of seeds of different *Stanleya* species collected in the field. (a) *S. pinnata* var. *integrifolia*; (b) *S. pinnata* var. *pinnata*; (c) *S. pinnata* var. *inyoensis*; (d) *S. bipinnata*; (e) *S. albescens*; (f) *S. viridiflora*; (g) *S. tomentosa*. Selenium (Se) distribution is shown in red, Ca in blue and Fe in green. Bars, 600μm.

Table 1 Selenium (Se) speciation in leaves of different *Stanleya* and *Thelypodium* species grown on Agar medium supplemented with 53 μM sodium selenate

Sample ID	NSS (10^{-4})	SeO_4	SeO_3	SeGSH2	SeCys	C–Se–C	Se^0
S. bipinnata	6.47	nd	9	nd	nd	91	nd
S. bipinnata	3.59	nd	0	nd	nd	99	nd
S. bipinnata	4.42	nd	nd	nd	nd	99	nd
Average		nd	5	nd	nd	96	nd
SD		nd	na	nd	nd	5	nd
S. elata 1	3.83	nd	2	nd	nd	96	nd
S. elata 1	4.82	nd	8	nd	nd	71	20
S. elata 1	2.92	nd	6	nd	nd	80	12
S. elata 2	8.07	7	11	nd	nd	79	nd
S. elata 2	7.72	9	11	nd	nd	78	nd
Average		8	8	nd	nd	80	16
SD		na	4	nd	nd	9	na
S. pinnata var. *integrifolia*	2.14	nd	5	nd	nd	82	14
S. pinnata var. *integrifolia*	6.5	nd	nd	14	nd	91	nd
S. pinnata var. *integrifolia*	7.41	nd	nd	nd	nd	96	nd
S. pinnata var. *integrifolia*	7.24	nd	nd	nd	nd	95	nd
S. pinnata var. *integrifolia*	5.78	2	nd	nd	nd	82	11
S. pinnata var. *integrifolia*	3.1	nd	nd	nd	nd	101	nd
S. pinnata var. *integrifolia*	4.8	nd	3	nd	nd	99	nd
Average		na	4	na	nd	92	12
SD		na	na	na	nd	8	na
S. pinnata var. *inyoensis* 1	6.33	nd	nd	nd	nd	97	nd
S. pinnata var. *inyoensis* 1	6.05	nd	nd	nd	nd	98	nd
S. pinnata var. *inyoensis* 1	4.26	nd	nd	nd	nd	98	nd
S. pinnata var. *inyoensis* 2	16.8	55	8	nd	nd	31	nd
S. pinnata var. *inyoensis* 3	3.73	nd	0	nd	nd	100	nd
S. pinnata var. *inyoensis* 3	8.39	nd	1	nd	37	59	nd
S. pinnata var. *inyoensis* 3	4.01	0	nd	nd	nd	99	nd
S. pinnata var. *inyoensis* 3	6.86	nd	nd	15	nd	82	nd
Average		na	3	na	na	83	nd
SD		na	3	na	na	25	nd
Sample ID	NSS (10^{-4})	SeO_4	SeO_3	SeGSH2	SeCys	C-Se-C	Se^0
S. pinnata var. *pinnata* 1	4.18	nd	nd	nd	nd	101	nd
S. pinnata var. *pinnata* 1	2.87	nd	nd	nd	nd	100	nd
S. pinnata var. *pinnata* 2	5.45	nd	nd	nd	nd	84	10
S. pinnata var. *pinnata* 2	2.09	nd	4	nd	nd	95	nd
Average		nd	na	nd	nd	96	nd
SD		nd	na	nd	nd	7	nd
S. tomentosa 1	6.18	nd	6	nd	nd	69	24
S. tomentosa 1	5.63	nd	nd	nd	nd	85	14
S. tomentosa 1	8.9	nd	nd	nd	nd	98	nd
S. tomentosa 2	9.29	nd	nd	nd	nd	95	nd
Average		nd	na	nd	nd	87	19
SD		nd	na	nd	nd	13	na
S. viridiflora	4.39	nd	nd	nd	nd	101	nd
S. viridiflora	14.2	nd	nd	nd	nd	102	nd
Average		nd	nd	nd	nd	101	nd
SD		nd	nd	nd	nd	na	nd
Th. laciniatum	12.8	nd	nd	nd	nd	97	nd
Th. laciniatum	23.1	nd	46	nd	nd	52	nd
Th. laciniatum	18.9	nd	25	nd	nd	72	nd
Th. laciniatum	16.8	nd	21	nd	nd	75	nd
Average		nd	31	nd	nd	67	nd
SD		nd	13	nd	nd	19	nd

Results from least squares linear combination fitting of experimental XANES spectra with standard selenocompounds. SeO_4, selenite; SeO_3, selenite; $SeGSH_2$, selenoglutathione; SeCys, selenocysteine; C–Se–C, methyl-selenocysteine/Se-Methionine (same spectra); Se^0, red or gray elemental Se; NSS, normal sum of squares (quality of fit; 0 = perfect fit); nd, compound not detected. Selenocystine was not detected in any sample. na, not applicable. Numbers following plant species names denote biological replicates. Spectra with identical numbers were collected at different positions on the sample.

S. pinnata var. *pinnata* and *S. tomentosa*) all had Se localized in patches in the margin of the leaf (Fig. 2). By contrast, *S. elata*, *S. pinnata* var. *inyoensis*, *S. viridiflora* and *Th. laciniatum* all had Se localized in the vascular tissue (Fig. 3). Field-collected seeds (Se concentrations reported earlier, Cappa *et al.*, 2014) from all species tested showed the same Se distribution. The Se was localized in the embryos (Fig. 4). Only *S. pinnata* var. *pinnata* had a slight Se signal in the seed coat. Selenium was not found in the endosperm of any species. Seeds from two of the species, *S. elata* and *Th. laciniatum*, did not have high enough Se concentrations to allow for X-ray microprobe analysis.

XANES

Of the species sampled here, grown in agar medium, we found > 50% organic Se in their leaf tissue, mainly modeled as C–Se–C compounds (Table 1). Note that the XANES spectra for selenomethione and methyl-selenocysteine are indistinguishable and thus the C–Se–C in these leaves and seeds can be a combination of the two compounds. The species with the marginal Se localization pattern generally also had a greater percentage of organic Se. The species with marginal Se distribution (*S. bipinnata*, *S. pinnata* var. *integrifolia*, *S. pinnata* var. *pinnata* and *S. tomentosa*) had, on average, 96%, 92%, 96% and 87% organic Se, respectively. The species with vascular Se localization (*S. elata*, *S. pinnata* var. *inyoensis*, *S. viridiflora* and *Th. laciniatum*) had 80%, 83%, 100% and 67% organic Se, respectively. The (inorganic) remainder of the leaf Se was selenate, selenite and elemental Se (Table 1). Regardless of plant species or the leaf localization and Se speciation we found the seeds to have almost exclusively organic Se, again mainly modeled as C–Se–C compounds; the remainder (inorganic) Se was mostly modeled as elemental Se (Table 2).

Table 2 Selenium (Se) speciation in seeds of different *Stanleya* taxa collected from the field

Sample ID	NSS (10^{-4})	SeO_4	SeO_3	C–Se–C	Se^0
S. albescens	2.46	<1	2	87	12
S. albescens	2.79	2	nd	88	11
S. albescens	4.76	nd	nd	100	nd
S. albescens	4.23	nd	1	100	nd
Average		1	2	94	12
SD		na	na	7	na
S. bipinnata	1.63	nd	nd	101	nd
S. bipinnata	1.52	nd	nd	93	8
Average		nd	nd	97	na
SD		nd	nd	na	na
S. pinnata var. *integrifolia* 1	4.43	1	nd	100	nd
S. pinnata var. *integrifolia* 1	3.59	nd	2	90	9
S. pinnata var. *integrifolia 1*	3.74	nd	<1	101	nd
S. pinnata var. *integrifolia 1*	2.44	nd	nd	100	nd
S. pinnata var. *integrifolia* 2	2.44	nd	nd	100	nd
Average		na	1	98	na
SD		na	na	4	na
S. pinnata var. *inyoensis 1*	5.03	1	nd	99	nd
S. pinnata var. *inyoensis 1*	4.88	nd	nd	101	nd
S. pinnata var. *inyoensis* 1	5.71	nd	nd	100	nd
S. pinnata var. *inyoensis* 2	2.2	nd	nd	102	nd
S. pinnata var. *inyoensis* 2	2.9	nd	nd	100	nd
Average		na	nd	100	nd
SD		na	nd	1	nd
S. pinnata var. *pinnata* 1	2.8	nd	nd	100	nd
S. pinnata var. *pinnata* 1	3.78	nd	<1	69	26
S. pinnata var. *pinnata* 2	2.86	nd	2	95	4
S. pinnata var. *pinnata* 2	3.56	1	nd	100	nd
S. pinnata var. *pinnata* 2	2.58	nd	<1	101	nd
S. pinnata var. *pinnata* 2	4.89	nd	nd	100	nd
S. pinnata var. *pinnata* 2	3.28	9	4	81	14
Average		5	2	92	15
SD		na	2	13	11
S. tomentosa	3.24	nd	nd	101	nd
S. viridiflora	3.29	nd	nd	101	nd

Results from least squares linear combination fitting of experimental XANES spectra with standard selenocompounds. SeO_4, selenite; SeO_3, selenite; $SeGSH_2$, selenoglutathione; SeCys, selenocysteine; C–Se–C, methyl-selenocysteine/Se-Methionine (same spectra); Se^0, red or gray elemental Se; NSS, normal sum of squares (quality of fit; 0 = perfect fit); nd, compound not detected. Additional standard compounds not detected in any sample: selenocystine, selenocysteine, selenoglutathione. na, not applicable. Numbers following plant species names denote biological replicates. Spectra with identical numbers were collected at different positions on the sample.

Phylogeny

Stanleya was not supported as monophyletic, as currently circumscribed (for data matrix and tree statistics, see Table 3). *Stanleya confertiflora* was resolved as sister to the clade of *Th. laciniatum* and *Th. ambigua* (Figs 5, S2). This result was recovered in the simultaneous analysis (Kluge, 1989) in both parsimony and likelihood as well as in two gene trees (LD and SAT; Fig. S2).The rest of *Stanleya* form a clade with 100% jackknife and 99% bootstrap support. Two main subclades were resolved within *Stanleya*: *Stanleya pinnata* (all varieties) and *S. bipinnata* constitute one highly supported subclade (99% jackknife, 93% bootstrap), whereas all other species were resolved as a separate subclade (< 50% jackknife, 91% bootstrap). *Stanleya tomentosa* and *S. viridiflora* were resolved as sister species in all analyses (Figs 5, S2). Within the *S. pinnata* clade, vars *integrifolia* and *texana* were resolved as exclusive lineages, each with > 50% jackknife support. *Stanleya bipinnata* was resolved as most closely related to two diploid *S. pinnata* var. *pinnata* accessions (both collected from the eastern slope of the Continental Divide). *Stanleya pinnata* var. *inyoensis* and one tetraploid *S. pinnata* var. *pinnata* (collected from the western slope of the Continental Divide) constitute another weakly supported clade. The remaining two *S. pinnata* var. *inyoensis* accessions also constitute a clade.

Ancestral reconstruction

Three *Stanleya* taxa (*S. bipinnata*, *S. pinnata* vars *integrifolia* and *pinnata*) have been documented as having Se concentrations of (or close to) > 1000 mg Se kg^{-1} DW in the field (Fig. 6a). Because these three taxa constitute a clade with *S. pinnata* var. *inyoensis* (Fig. 5), the origin of hyperaccumulation is ambiguously optimized for the *S. bipinnata/pinnata* clade after collapsing clades with < 50% jackknife and bootstrap support (Fig. 6a). If

Table 3 Data matrix and tree statistics for each of the phylogenetic analyses

Matrix	No.of terminals	No.of Characters analyzed	No.of parsimony informative characters	% Missing/ inapplicable	Most parsimonious tree length	No.of most parsimonious trees	No.of jackknife/ bootstrap clades ≥ 50%	Average jackknife/ bootstrap support (%)	CI	RI
CHS	35	824	46	3.5	163	3296	8/9	77/55	0.97	0.98
ITS	39	713	62	7.6	211	21 202	9/11	94/58	0.71	0.87
LD	32	626	76	12.1	173	9	12/9	82/79	0.91	0.96
SAT	33	621	40	11.6	131	56	14/11	78/60	0.88	0.95
All molecular	39	2784	224	17.7	696	297	15/17	75/74	0.8	0.9
Morphological	14	15	14	0.1	42	6	1	57	0.73	0.71
Simultaneous	39	2799	238	17.6	745	3	17	64	0.78	0.9

CHS, chalcone synthase; ITS, internal transcribed spacer; LD, luminidependens; SAT, nongenic region; CI, ensemble consistency index (Kluge & Farris, 1969) on the most parsimonious tree(s) for the parsimony-informative characters; RI, ensemble retention index (Farris, 1989).

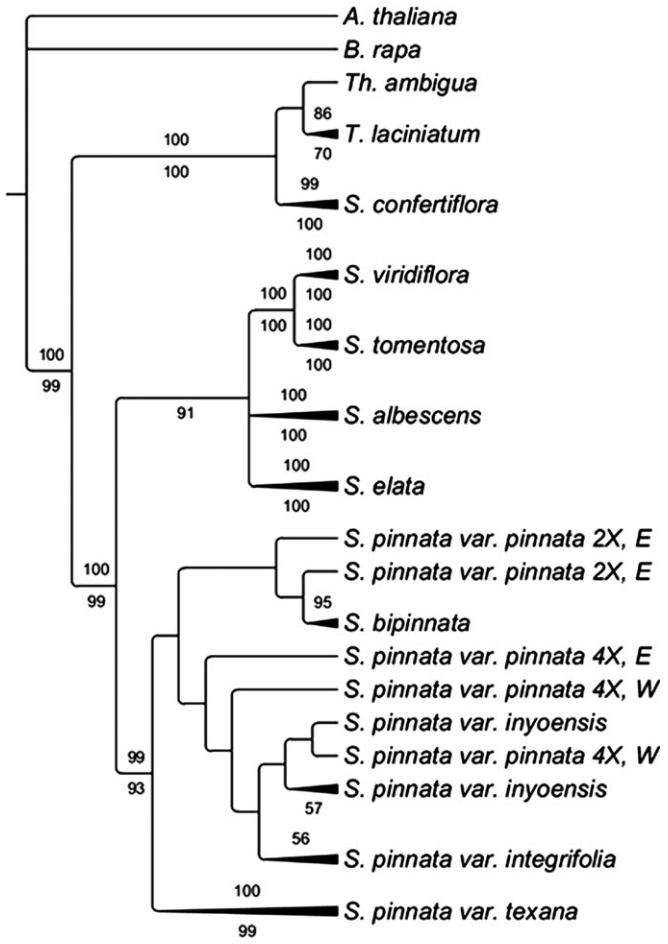

Fig. 5 Strict consensus of three most parsimonious trees for the simultaneous analysis of both molecular and morphological data. Clade symbols represent 2–4 individuals per taxon. Values above and below the branches represent parsimony jackknife and likelihood bootstrap support values ≥50%, respectively. Values next to *Stanleya pinnata* var. *pinnata* accessions indicate diploid (2x) or tetraploid (4x) and collection site east slope (E) or west slope (W). *A. thaliana*, *Arabidopsis thaliana*; *B. rapa*, *Brassica rapa*; *Th. ambigua*, *Thelypodiopsis ambigua*; *T. laciniatum*, *Thelypodium laciniatum*.

S. pinnata var. *texana* is indeed the sister group of the remaining members of *S. pinnata* and *S. bipinnata* (Fig. 5), then the most parsimonious inference is that selenium hyperaccumulation evolved within the clade after the divergence of *S. pinnata* var. *texana*. By contrast, the clade comprising the rest of *Stanleya* is not inferred to have had a hyperaccumulator ancestor (Fig. 6a).

Based on the results from our agar experiment, most of the taxa we sampled are tolerant to hyperaccumulator Se concentrations (> 1000 mg kg^{-1} DW; Fig. 6b); Se-sensitive exceptions include the most distant outgroup (*A. thaliana*) as well as the *S. viridiflora/S. tomentosa* clade and *S. pinnata* var. *inyoensis*. Three taxa showed no significant growth reduction by Se, at tissue Se concentrations upwards of 2000 mg kg^{-1} DW. These include the Se hyperaccumulators *S. pinnata* var. *pinnata* and *S. bipinnata,* as well as *S. albescens*. Thus, Se tolerance at the ≥ 1000 mg kg^{-1} DW concentration is unambiguously optimized as the ancestral state for the entire *Stanleya/Thelypodium/Brassica* clade. There appears to be an additional amount of hypertolerance at the 2000 mg kg^{-1} DW concentration in the two most extreme Se hyperaccumulator taxa (*S. bipinnata* and *S. pinnata* var. *pinnata*). From these combined ancestral reconstructions of Se tolerance and accumulation we infer that Se tolerance evolved before Se accumulation in *Stanleya*.

Discussion

The primary question addressed in this study is: what is the evolutionary history of Se hyperaccumulation in *Stanleya*? This includes: how many times has hyperaccumulation evolved and been lost, and did hyperaccumulation and tolerance evolve simultaneously in *Stanleya* or did one precede the other? Our results show that Se hyperaccumulation (> 1000 mg Se kg^{-1} DW *in situ*) is restricted to the *S. bipinnata/pinnata* clade (Fig. 6a). Based on the collapsed tree shown in Fig. 6(a), there are two alternative parsimony reconstructions, each with three steps. Either hyperaccumulation evolved on the branch leading to the *S. bipinnata/pinnata* clade and was lost independently in *S. pinnata* vars *inyoensis* and *texana*, or there were three independent origins of hyperaccumulation in *S. bipinnata* and *S. pinnata* vars *integrifolia* and *pinnata*. To fully answer this question requires full resolution of the relationships within the *S. bipinnata/pinnata* clade. Based on the relationships within that clade shown in Fig. 5, wherein *S. pinnata* var. *texana* is sister to the remaining members of this clade, hyperaccumulation is most parsimoniously inferred to have

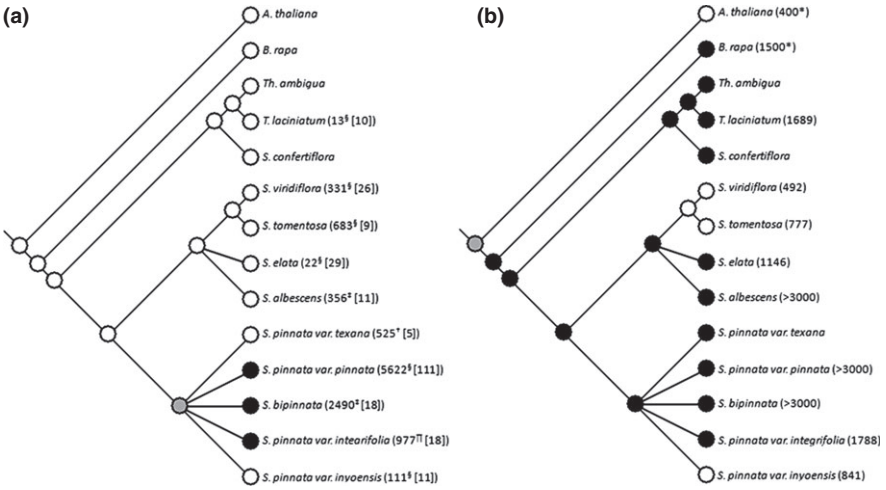

Fig. 6 Selenium (Se)-related traits mapped onto the collapsed, simultaneous-analysis phylogeny using parsimony. (a) Accumulation (maximum field Se concentration (n)); (b) tolerance (50% inhibition as mg Se kg^{-1} DW). Light gray circles, ambiguous; white circles, unknown, nonhyperaccumulator and sensitive; black circles, hyperaccumulator and tolerant. Estimated from *Garifullnia *et al.* (2003), Van Huysen *et al.* (2003) and Zhang *et al.* (2007); §Cappa *et al.* (2014); †Beath *et al.* (1939b); ‡Beath *et al.* (1940); ∏Beath *et al.* (1941). *A. thaliana*, *Arabidopsis thaliana*; *B. rapa*, *Brassica rapa*; *Th. ambigua*, *Thelypodiopsis ambigua*; *T. laciniatum*, *Thelypodium laciniatum*; *S.*, *Stanleya* species.

had a single origin within this clade and was followed by one loss in *S. pinnata* var. *inyoensis*. Among the non-Se hyperaccumulator taxa, all but one are classified as secondary Se accumulators based on the maximal field Se concentrations reported (> 100 mg kg^{-1} DW, Fig. 6a). *Stanleya elata* is classified as a non-Se accumulator, together with outgroup *Th. laciniatum*. The finding that there is a large intraspecific variation in Se hyperaccumulation and hypertolerance within *S. pinnata* is in agreement with earlier reports by Feist & Parker (2001). Similar intraspecific variation has been reported for Zn and Cd hyperaccumulation in *A. halleri* (Koch & German, 2013).

Tolerance to Se occurs broadly in the *Stanleya/Thelypodium/Brassica* clade (Fig. 6b). The ancestral reconstruction of tolerance, as scored here, unambiguously indicates that tolerance evolved before hyperaccumulation in *Stanleya*. A similar pattern was found for Zn hyperaccumulation and tolerance in *Noccaea*: high shoot Zn concentrations were found throughout *Noccaea* and its sister genus *Raparia* but not in *Thlaspiceras*, which nevertheless displays Zn hypertolerance (Peer *et al.*, 2003; Broadley *et al.*, 2007; Koch & German, 2013). Based on our results, tolerance to tissue Se concentrations above 1000 mg kg^{-1} DW is more prevalent in *Stanleya* than the capacity to actually attain these hyperaccumulator concentrations in the field. While Se tolerance may be a prerequisite for hyperaccumulation, it is not always predictive of it. Based on this reconstruction, we infer that the *S. viridiflora/tomentosa* clade lost tolerance to high Se concentrations. Interestingly, in both of these species the maximum field Se concentration recorded was just below their 50% inhibition concentration. Also, these two species' geographical distributions appear to correspond to areas with low soil Se concentrations (Cappa *et al.*, 2014). It is feasible that Se hypertolerance has a fitness cost, and therefore is lost on soils with low Se concentrations, in analogy with reports of a fitness cost for Zn hypertolerance on low-Zn soil in *Silene* (Broadley *et al.*, 2007).

Our inferences about Se tolerance concentrations are based on the agar study presented here (Fig. 1), which currently is the only common-garden experiment where the growth of most *Stanleya* taxa (excluding *S. confertiflora* and *S. pinnata* var. *texana*) were compared in the presence and absence of Se. While agar experiments are commonly used to assess the tolerance index to toxic elements, it is possible that alternative experimental systems (e.g. on hydroponics or soil) or testing more mature plants may give different results. For instance, in one of our earlier studies *S. albescens* was significantly less tolerant to Se than *S. pinnata* var. *pinnata* (Freeman *et al.*, 2010). Moreover, growth of *S. elata* has been shown in our earlier studies to be 50% inhibited at tissue Se concentrations well below 1000 mg kg^{-1} DW, which would classify it as Se sensitive in this study (El Mehdawi *et al.*, 2012; Lindblom *et al.*, 2014).

The main tolerance mechanism in Se hyperaccumulators is to store Se in the form of nonprotein amino acids, as reviewed in the introduction. Indeed, the Se hyperaccumulator taxa all stored Se in the form of organic C–Se–C compounds (Tables 1, 2). The outgroups, *A. thaliana* and *B. juncea*, were shown in earlier studies to accumulate predominantly inorganic selenate when supplied with selenate (de Souza *et al.*, 1998; Van Hoewyk *et al.*, 2005; Freeman *et al.*, 2006). A close relative of *Stanleya*, *Th. laciniatum*, had the lowest percentage of organic Se (65%) for all species tested here. All *Stanleya* taxa tested accumulated at least 80% of Se in the form of C–Se–C compounds. This suggests that *Stanleya* has evolved an increased capacity to convert selenate to organic C–Se–C, as compared with related genera. Despite the finding that all tested *Stanleya* taxa contained predominantly C–Se–C compounds, they showed variation in Se tolerance. The reason for this variation could be that they contained different C–Se–C compounds. In *S. albescens*, for instance, the C–Se–C was found to be selenocystathionine, while in *S. pinnata* var. *pinnata* it was methyl-selenocysteine (Freeman *et al.*, 2006, 2010). An additional Se tolerance mechanism in hyperaccumulators may be specialized sequestration in peripheral leaf cells: three of the four species that showed this Se localization pattern were highly Se tolerant and reached hyperaccumulator concentrations in the field. Similarly, Se hyperaccumulator *A. bisulcatus* was found to sequester Se mainly in its leaf periphery, in trichomes (Freeman *et al.*, 2006) and Zn hyperaccumulator *Noccaea caerulescens* predominantly stores Zn in the vacuoles of epidermal cells (Vázquez *et al.*, 1994). Overall, the results from these XAS studies indicate that production of organic Se compounds and specialized

sequestration are not necessarily predictive of tolerance or hyperaccumulation, but may be steps in the evolution of Se hyperaccumulation, and perhaps a prerequisite.

In the ancestral reconstruction of Se hyperaccumulation and tolerance discussed above, *S. pinnata* var. *pinnata* was treated as a single lineage, because there was < 50% support for resolution of the different accessions (Fig. 5). As described earlier (Cappa *et al.*, 2014), *S. pinnata* var. *pinnata* contains both diploid and tetraploid accessions, with all diploids occurring east of the Continental Divide and all but one tetraploid accession occurring west of the Continental Divide. Hyperaccumulation was found exclusively in diploid accessions, with one exception: a tetraploid found close to the lowest point of the Continental Divide (Great Divide Basin, WY, USA). It is intriguing that there are two ploidy levels in *S. pinnata*, and tempting to hypothesize that ploidy levels correlate with hyperaccumulation capacity. However, when tested under controlled conditions, *S. pinnata* var. *pinnata* can reach hyperaccumulator concentrations regardless of its ploidy level or geographic origin (Harris *et al.*, 2014; Fig. S3). In addition, all *S. pinnata* varieties tested appear to have hyperaccumulation capacity when provided with selenate in a controlled environment (Fig. S3); however, whether these taxa actually hyperaccumulate in the field appears to depend not only on this innate capacity, but also on the environment. A similar division may be the case for *S. bipinnata*. Beath *et al.* (1940) reported *S. bipinnata* with tissue Se concentrations of up to 2490 mg Se kg^{-1} DW in a population outside Laramie, WY, USA, on the eastern side of the Continental Divide. Unfortunately the population does not exist any more due to development, and could not be resampled for our study. All of the *S. bipinnata* collected by Cappa *et al.* (2014) were west of the Continental Divide, and none reached hyperaccumulator concentrations *in situ*. Nevertheless, these accessions did have hyperaccumulation capacity when provided with selenate in a controlled environment (Fig. 1).

The uncollapsed phylogeny of *Stanleya* (Fig. 5) resolves *S. bipinnata* nested within *S. pinnata*, and most closely related to the Se hyperaccumulating diploids of *S. pinnata* var. *pinnata*. Thus, *S. bipinnata* may actually be a variety of *S. pinnata* as asserted by Rollins (1939). If this clade continues to be supported in a more extensive phylogenetic analysis, with increased character sampling, we hypothesize that Se hyperaccumulation evolved within this clade, after the divergence of *S. pinnata* var. *texana* (which is highly supported as distinct from all other member of *S. pinnata*). The other subclade within the *S. pinnata/bipinnata* clade (Fig. 5) is composed of all *S. pinnata* tetraploid accessions, including the tetraploid lineages of *S. pinnata* var. *pinnata*, and vars. *integrifolia* and *inyoensis*. The 2x and 4x *S. pinnata* var. *pinnata* accessions are separated from each other into two sister clades and could be considered separate species because they are reproductively isolated owing to a postzygotic barrier. Another taxonomic consideration from this study is that *S. confertiflora* is clearly not a member of *Stanleya* s.s. Based on our sampling, it cannot be determined which other genus it should be classified as; this will require more study. Cacho *et al.* (2014) showed two species of *Stanleya* (*S. pinnata* and *S. elata*) to be nested within *Streptanthus* and sister to species of *Caulanthus*. The monophyly of *Stanleya*, as resolved here, needs to be more rigorously tested, with greater taxonomic sampling, across the Thelypodieae.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Selenium and sulfur concentrations in shoots of different *Stanleya* and *Thelypodium* species, supplemented with different concentrations of sodium selenate.

Fig. S2 Strict consensus of most parsimonious trees for molecular data.

Fig. S3 Leaf Se accumulation in *Stanleya pinnata* varieties and ploidy variants.

Table S1 List of taxa sampled with taxonomic authorities, accession information, herbarium voucher information, and TAIR and GenBank accession numbers for new sequences generated for this study

Table S2 Morphological characters and chromosome numbers used in phylogenetic analysis

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